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Studies on the New Metabolic Pathway of Anthranilic Acid in the Rat. I. Isolation and Urinary Excretion of Anthranilamide as a New Metabolite of Anthranilic Acid[1]

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(1) A new metabolite was isolated in crystalline form from urine of rats injected with anthranilic acid.

(2) This compound was identified as anthranilamide by chemical and physical analyses.

(3) It has been found that the urine from rats injected with anthranilic acid contained anthranilamide in the range of 1.2—2.5%.

(4) It was found that a large part of anthranilamide was transformed into other metabolites, and onlu 5% of the dose could be found as free anthranilamide.

(5) The possible new metabolic pathway of anthranilic acid was discussed.

Anthranilic acid as a metabolite of tryptophan has been extensively studied in different animals by many investigators. In mammalian animals, anthranilic acid is acetylated on the amino group and conjugated with glycine or glucuronic acid at the carboxyl group. Conjugation of anthranilic acid with glycine was reported by Mason,[3] and that with glucuronic acid was first demonstrated by Mitsuba and Ichihara,[4] and they[5] further showed that administration of anthranilic acid to dogs and rabbits increased urinary excretion of reducing substances and ethereal sulphates. They concluded that this conjugation was a part of the detoxication mechanism in the body.

On the other hand, Knoefel, *et al.*[6] stated that in both species the rate of conjugation with glycine or glucuronic acid was the highest in *o*-aminobenzoic acid (anthranilic acid) among three isomers of *o*-, *p*-, and *m*-aminobenzoic acids.

Metabolism of anthranilic acid has also been investigated thoroughly in vertebrates and insects, in which formation of anthranylglucuronide and *o*-aminobenzoylglycine was found by Jaffe[7] and Takahashi.[8] They also confirmed the formation of ornithuric acid in the hen and of ornithine conjugate in a 14-day-old chick embryo after inoculation with benzoic acid.

Tabone[9] has reported that β-glucoside of anthranilic acid was converted into anthranilamide in the presence of ammonia at room temperature, and Jacobelli and Tabone[10] have found anthranilamide in the medium of *Escherichia coli* was added with anthranilic acid.

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1) This work was presented in part at the Meeting of the Japanese Biochemical Society in Hiroshima, October 1969 (T.M. Sutamihrdja, A. Ishikura, J.Naito, and I. Ishiguro, *Seikagaku*, **41**, 486 (1969)).
2) Location: *492-36 Mitahora, Gifu, 501-21, Japan.*
3) M. Mason, *J. Biol. Chem.*, **201**, 513 (1953).
4) K. Mitsuba and K. Ichihara, *Osaka Med. Mitt.*, **25**, 1813 (1926).
5) K. Mitsuba and K. Ichihara, *Hoppe- Seyler's Z. Physiol. Chem.*, **164**, 244 (1927).
6) P.K. Knoefel, K.C. Huang, and A. Despopolous, *Am. J. Physiol.*, **196**, 1224 (1959).
7) M. Jaffe, *Ber. Dtsch. Chem. Ges.*, **10**, 1925 (1877).
8) M. Takahashi, *J. Physiol. Chem.*, **6**, 291 (1928).
9) J. Tabone, *Bull. Soc. Chim. Biol.*, **43**, 1221 (1961).
10) G. Jacobelli and J. Tabone, *Bull. Soc. Chim. Biol.*, **43**, 1197 (1961).

Recently, Kutacek and Galston[11] showed that anthranilic acid was converted into anthranyl-β-glucoside, tryptophan, kynurenine, and a small amount of anthranilamide in green and etiolated pea stem.

Since there is no report on the formation of anthranilamide from anthranilic acid in mammalians, we examined the metabolism of anthranilic acid in more detail.

This paper describes the isolation and identification of anthranilamide excreted in the urine after administration of anthranilic acid to the rats, suggesting a possible new metabolic pathway of anthranilic acid.

Experimental

Materials and Methods——a) Materials: Pure anthranilic acid and anthranilamide were purchased from the Wako Pure Chemical Industries Ltd., Tokyo. Just before injection into rats, anthranilic acid was dissolved in 1N NaOH, neutralized with 1N HCl, and the solution injected into the experimental animals. Synthetic anthranilamide was dissolved in saline solution before use.

b) Apparatus: The experimental animals were housed in a metabolic cage and their urine was collected in a cylinder layered with toluene. Ultraviolet (UV) spectra were determined with a Hitachi model MPS recording spectrophotometer and the infrared (IR) spectra were recorded with a Perkin-Elmer model 22 spectrophotometer. Free anthranilic acid and anthranilamide were measured according to the modification of Bratton-Marshall method,[12] using a Hitachi model 101 spectrophotometer.

c) Animals: Male albino rats of the Wistar strain were maintained on CE-2 pellet diet (Oriental Yeast Co., Tokyo), and the rats weighing 150—200 g were used as the experimental animals.

Anthranilic acid was injected intraperitoneally into the experimental rats at a dosage of 100 mg and its metabolites were isolated from urine. To study the effect of dosage on the urinary excretion of its metabolites, anthranilic acid was injected intraperitoneally into rats at dosages of 1, 10, 50, or 100 mg/rat or anthranilamide 10 or 50 mg/rat.

d) Collection of Urine: Urine samples from rats injected with anthranilic acid or anthranilamide were collected daily into a cylinder layered with toluene and kept frozen until use. Urine collected for 24 hr was filtered through glass wool and was supplied for further experiment or determination of anthranilamide.

e) Paper Chromatography: Urinary metabolites were identified by one- or two-dimensional ascending chromatography on Toyo Roshi No. 51 filter paper, using BuOH–AcOH–H_2O (4:1:1) and PrOH–EtOH–H_2O (2:1:1) as solvents, and were allowed to develope for 16 hr.

Urine collected for 24 hr from rats injected with anthranilic acid or anthranilamide was filtered and then concentrated to dryness under a reduced pressure. The residue was dissolved in 70% EtOH, centrifuged, and the supernatant was evaporated into a small volume for chromatographic analysis.

f) Chemical and Physical Analyses of Isolated Metabolite: The isolated unknown metabolite was identified by paper chromatography, UV and IR spectra, various color reactions,[13] elemental analysis, and comparison of its hydrolysis product with authentic anthranilamide.

g) Determination of Anthranilic Acid, Anthranilamide, and Their Conjugated Type: One ml of urine from a rat injected with anthranilic acid was used for the determination of free anthranilic acid and free anthranilamide through the modification of the Bratton–Marshall method.[12] Free anthranilamide was determined by extracting it 3 times with 10 ml each of ether from alkaline urine in 0.1N NaOH (1 ml of urine, 8 ml of distilled water, and 1 ml of 1N NaOH). Free anthranilic acid was determined by extracting it 3 times with 10 ml each of ether from the aqueous layer adjusted to pH 3.0 with HCl. The glucuronide type was estimated after hydrolysis of the urine in 0.1N NaOH (final) at 55° for 1 hr. The acetylated type was estimated by hydrolysis with 1N HCl in a boiling water for 1 hr, and the strongly conjugated type (peptide) by hydrolysis in 6N HCl for 1 hr in a sealed tube at 110°.

Result

Paper Chromatographic Separation of Urinary Metabolites from Rats injected with Anthranilic Acid

Many known metabolites of anthranilic acid and related substances in urine from rats injected with anthranilic acid showed strong fluorescence with considerable variation in color. As shown in Fig. 1, by two-dimensional paper chromatography using BuOH–AcOH–H_2O

11) M. Kutacek and A.W. Galston, *Plant Physiol.*, **43**, 1793 (1968).
12) A.C. Bratton and J.K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537 (1937).
13) C.E. Dalgliesh, *Biochem. J.*, **52**, 1 (1951).

(4:1:1) and PrOH–EtOH–H_2O (2:1:1) as solvents, 12 strongly fluorescent spots were observed under ultraviolet ray. All these spots may be metabolites of anthranilic acid and some of them were characterized as follows: Spot A was free anthranilic acid, as nonmetabolized anthranilic acid, spot B with a light blue fluorescence was 2-methyl-1,2,3,4-tetrahydro-4-quinazolone which had been identified in our laboratory,[14] spot C with violet fluorescence which we designated as "V substance" is an unknown metabolite at present, spot D and spot E with strong violet fluorescence have been known as the two main metabolites of anthranilic acid. Spot D was *o*-aminobenzoylglycine, and spot E was anthranylglucuronide. Other spots have not been identified yet.

Purification of V Substance

A method for purification of the V substance from the urine of rats injected with anthranilic acid is shown in Chart 1. One liter of urine collected from 100 rats after injection of 100 mg of anthranilic acid per rat was made alkaline with 0.1N NaOH and then extracted

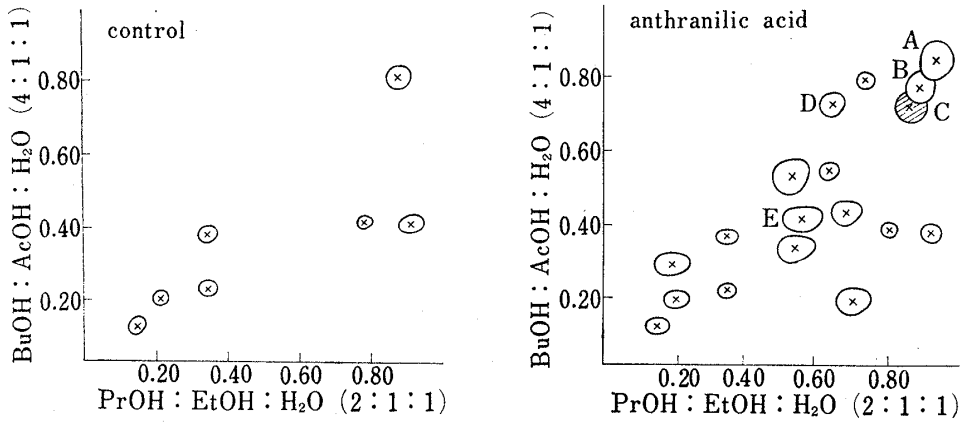


Fig. 1. Two-dimensional Paper Chromatography of Urinary Metabolites of Anthranilic Acid

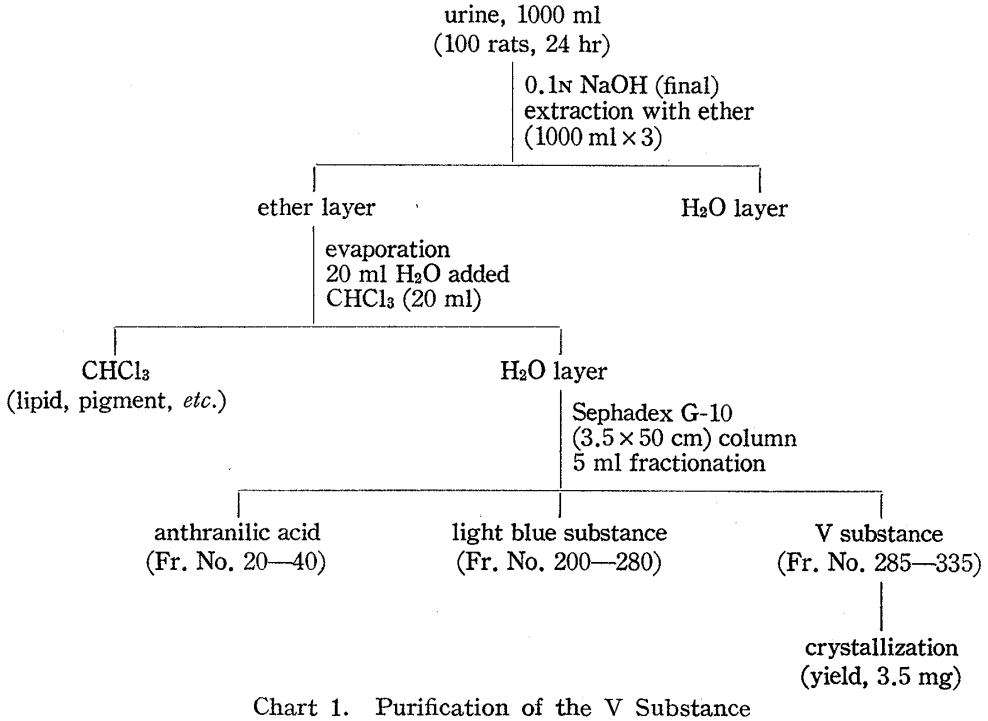


Chart 1. Purification of the V Substance

14) A. Ishikura, J. Naito, and I. Ishiguro, Abstr. Papers, 90th Annual Meeting of Meet. Pharm Soc. Japan, III-218 (1970).

3 times with 1 liter each of ether. Almost all the free anthranilic acid and V substance transited into the ether layer. Ether layer was evaporated to dryness, the residue was dissolved in 20 ml of water, washed twice with 20 ml of $CHCl_3$ to remove contaminated pigment (urochrome) and lipids, and the aqueous solution was applied on a column (3.5×50 cm) of Sephadex G-10 that had been washed with water. The column was eluted with water at a flow rate of 30 ml/hr, and the eluate was collected in 5-ml fractions. Each fraction was assayed for fluorescence under UV ray. Free anthranilic acid (fraction No. 20—40) and light blue substance (2-methyl-1,2,3,4-tetrahydro-4-quinazolone) (fraction No. 200—280) were separated from the V substance. Fractions containing V substance (Fraction No. 285—335) were combined and evaporated to dryness *in vacuo*. The residue was dissolved in a small amount of EtOH and the V substance crystallized when the EtOH solution was kept in an ice box for 24 hr. Recrystallization was carried out in the same way and 3.5 mg of pure crystalline V substance was obtained.

Chemical Properties and Identification of V Substance

The *Rf* values of the isolated V substance were compared with authentic anthranilamide after chromatographic development on a filter paper in various solvents. Both authentic anthranilamide and V substance gave an *Rf* value of 0.73 in a solvent system of BuOH–AcOH–H_2O (4: 1: 1) and an *Rf* 0.88 in PrOH–EtOH–H_2O (2: 1: 1). Spots of V substance and authentic anthranilamide on the filter paper were subjected to color reactions of Bratton–Marshall, Ninhydrin, aldehyde (Ehrlich's reagent), $FeCl_3$, $KMnO_4$, and $AgNO_3$-ammonia reagents. V substance and authentic anthranilamide gave the same color reactions on a filter paper. Both were positive to the tests for Bratton–Marshall, aldehyde, and $KMnO_4$ reactions.

The absorption spectrum of the isolated V substance in H_2O had two peaks at 243 and 420 nm which were identical with those of authentic anthranilamide (Fig. 2).

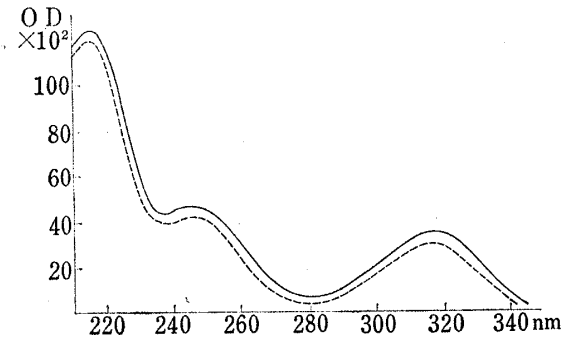


Fig. 2. Ultaviolet Absorption Curves of the Isolated V Substance (———) from Urine and Authentic Anthranilamide (--------) measured in Distilled Water

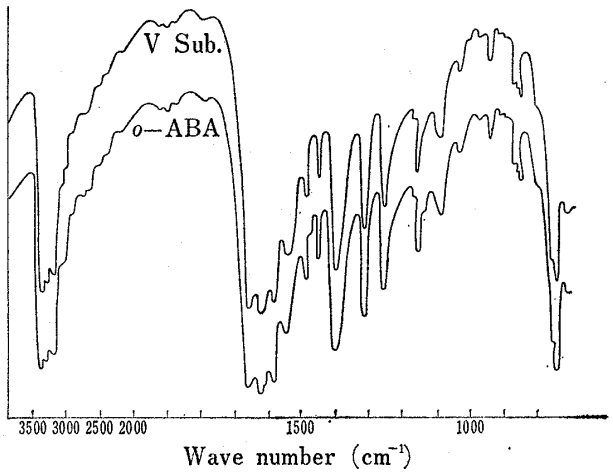


Fig. 3. The Infrared Spectra of V Substance and the Authentic Anthranilamide (*o*-ABA)

IR spectra of both V substance and the authentic anthranilamide also showed the same absorption bands at 3540, 3420, and 1661 cm^{-1} as shown in Fig. 3. The absorption band at 1661 cm^{-1} seems to correspond to the aromatic carbonyl group with an amino group in the *ortho* position, and the bands near 3420 and 3540 cm^{-1} suggest the presence of an amino and amido group.

The V substance or anthranilamide was stable on boiling for 1 hr in 1N HCl or 1N NaOH, and gave one spot with an *Rf* 0.70 on paper chromatogram with a solvent of NH_3–EtOH–H_2O (1: 18: 1), but it was unstable on heating at 110° in 6N HCl for 2 hr, being converted into

a substance with an *Rf* value of 0.35, corresponding to anthranilic acid. It is likely that the V substance is anthranilamide and was hydrolysed to anthranilic acid.

The V substance occurred as leaflet crystals and melted at 110° (decomp.) and showed violet fluorescence on the paper chromatogram under the UV ray, showing the same properties as the authentic anthranilamide (*Anal.* Calcd. for $C_7H_8ON_2$ (anthranilamide): C, 61.75; H, 5.92; N, 20.57. Found: C, 61.60; H, 5.96; N, 20.83). From these results, the V substance, excreted in urine after injection of anthranilic acid into rats, was identified as anthranilamide.

Determination of Urinary Anthranilamide in Rats injected with Anthranilic Acid

Urinary metabolites were determined after intraperitoneal injection of varied amount of anthranilic acid (1 to 100 mg) into the experimental animals. Percentage of urinary excretion of free anthranilic acid decreased from 70 to 50% when the dosage of the injected anthranilic acid was increased from 1 to 100 mg (Table I), and the percentage of urinary excretion of free anthranilamide increased from 1.2 to 2.5% (w/w).

Table I. Determination of Urinary Anthranilamide in Rats injected with Anthranilic Acid

Dose (mg)	a Anthranilic acid		b Anthranilamide		(b/a) × 100
	(mg/day)	(%)	(mg/day)	(%)	
1	0.733	73.3	0.012	1.2	1.6
10	6.847	68.5	0.200	2.0	2.9
50	34.983	70.0	1.243	2.5	3.5
100	49.752	49.8	1.863	1.9	3.8

The values are the average of 3 animals, and are corrected from the control.

As shown in Table II, only 1.73% of 10 mg of anthranilamide injected was excreted as free anthranilamide, and about 1.08% as free anthranilic acid. When the dosage was increased to 50 mg, 4.8% was excreted as free anthranilamide and 0.44% as free anthranilic acid.

Table II. Determination of Urinary Anthranilamide and Anthranilic Acid in Rats injected with Anthranilamide

Dose (mg)	Anthranilamide		Anthranilic acid	
	(mg/day)	(%)	(mg/day)	(%)
10	0.173	1.73	0.108	1.08
50	2.416	4.80	0.218	0.44

The values are the average of at least 3 animals.

Table III. Determination of Urinary Anthranilic Acid and Its Conjugates in Rats injected with 100 mg of Anthranilic Acid

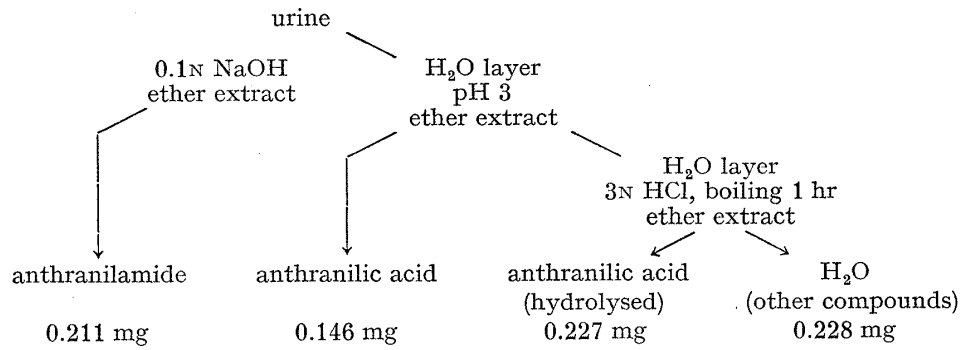
	Injected	Control
	(mg/rat/day)	
Free type	38.80	0.03
Glucuronide	4.30	0.02
Acetylated	0.73	0.10
Peptide	2.04	0.03

The values are the average of at least 3 animals.

As shown in Table III, after injection of anthranilic acid at the dosage of 100 mg/rat, about 40% was excreted into urine as free type, about 9% as conjugate type, 4.3% as glucuronide type, 0.8% as acetylated type, and 3% as the peptide type (glycine conjugate).

Table IV shows the results of the estimation of urinary metabolites for 24 hr after administration of anthranilamide at the dosage of 10 mg/rat. About 0.2 mg of anthranilamide, 0.15 mg of free anthranilic acid, and 0.45 mg of conjugate were found. The total of the excreted products corresponded to about 8% of the administered anthranilamide.

Table IV. Determination of Urinary Anthranilamide and Its Conjugate in Rats injected with 10 mg of Anthranilamide



Discussion

Several main metabolites of anthranilic acid were examined by paper chromatography of urine obtained during 24 hr after intraperitoneal injection of anthranilic acid into rats. These metabolites were anthranylglucuronide, a conjugate of anthranilic acid with glucuronic acid, *o*-aminohippurate, a conjugate of anthranilic acid with glycine, and also two new metabolites which we have identified. One of them was 2-methyl-1,2,3,4-tetrahydro-4-quinazolone,[14] which had been identified in our laboratory, and the other was characterized as anthranilamide from the results of elementary analysis, color reaction, and IR spectra.

The occurence of anthranylglucuronide and *o*-aminohippurate has already been reported,[3,4] but up to now there was no report on the formation of anthranilamide after administration of anthranilic acid to higher animals, although Tabone,[9] and Jacobelli and Tabone,[10] reported that anthranilamide could be formed from β-glucoside of anthranilic acid during their studies on *Escherichia coli*. To make certain whether urinary anthranilamide is an artifact product or the metabolic product of administered anthranilic acid, extensive *in vivo* studies were conducted in our laboratory.

It is clear from our results[15] that a part of anthranilic acid injected into rats was metabolically converted into anthranilamide, and the possibility of the artifact formation of anthranilamide in urine was excluded.

Quantitative studies on urinary anthranilamide after dosing varied amount of anthranilic acid (Table I) showed that the excretion of anthranilamide was relatively small and was in the range of 1 to 2% of the dose.

On the other hand, when anthranilamide was injected at the dosage of 10 mg, only 0.17 mg or 1.73% of the dose of free anthranilamide could be found in urine. When the dosage of anthranilamide was increased to 50 mg, 2.4 mg, or 4.8% was found as free anthranilamide.

Paper chromatographic examination showed, anthranilamide was transformed into several metabolites in the body, mainly into two metabolites which gave blue and violet fluo-

15) I. Ishiguro, J. Naito, T.M. Sutamihardja, and A. Ishikura, unpublished.

rescence. The isolation and identification of these two substances as new metabolites of anthranilamide will be reported elsewhere.

Metabolic fate of injected anthranilamide seems to be different among mammalian species. As reported by Bray, *et al.*,[16] in a rabbit, about 30% of the administered anthranilamide was found as the ether soluble material and 20—40% was as conjugated form of anthranilamide.

It is clear from paper chromatographic and quantitative studies that a large part of injected anthranilamide was transformed into some metabolites.

The metabolic relationship between anthranilic acid and anthranilamide must be clarified in future, but there is a possibility that anthranilamide is an intermediate metabolite of anthranilic acid, or one of the intermediates of tryptophan metabolism.

16) H.G. Bray, H.J. Lake, F.C. Neale, W.V. Thorpe, and P.B. Wood, *Biochem. J.*, **42**, 434 (1948).